

1 **Genome-wide association study and heritability estimate for ectopic ureters in**
2 **Entlebucher mountain dogs**

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20 **Abstract**

21

22 An ectopic ureter is a congenital anomaly which may lead to urinary incontinence and
23 without a surgical intervention even to end-stage kidney disease. A genetic component
24 contributes to the development of this anomaly in Entlebucher mountain dogs (EMD).
25 However, its nature remains unclear. Using the Illumina CanineHD bead chip, a case-control
26 genome-wide association study was performed to identify SNPs associated with the trait.
27 Six loci on canine chromosomes 3, 17, 27 and 30 were identified with 16 significantly
28 associated SNPs. There was no single outstanding SNP associated with the phenotype and
29 the association signals were not close to known genes involved in human congenital
30 anomalies of the kidney or lower urinary tract. Additional research will be necessary to
31 elucidate the potential role of the associated genes in the development of ectopic ureters in
32 the EMD breed.

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36 **Keywords:** GWAS, urinary tract development, *Canis lupus familiaris*, dog, CAKUT,
37 CALUT, Entlebucher mountain dog

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46 In dogs, an ectopic ureter (EU) is a rare congenital anomaly in which one or both ureteral
47 orifices are not located at the anatomically correct position at the vesicular trigone of the
48 bladder (Osborne *et al.* 1995). Instead, the ureteral openings can be found in the bladder
49 neck, the urethra or even in the genital system (Dean 1988; Owen 1973). The most common
50 clinical sign of affected dogs is urinary incontinence (Fossum 1997). However, many dogs,
51 especially males, which show a higher prevalence for EU than females in the Entlebucher
52 mountain dog (EMD) (Fritsche *et al.* 2014), are continent for years and may show
53 incontinence only at an advanced age (Holt & Moore 1995; Reichler *et al.* 2012). Affected
54 dogs are predisposed for ascending infections of the urinary tract including pyelonephritis;
55 they may have or develop hydroureter and hydronephrosis, as well as changes of kidney
56 size or distorted internal kidney architecture (Holt & Moore 1995; Lamb & Gregory 1998;
57 Niesterok 2016). This may result in fatal end-stage kidney disease if no surgery is performed.
58 The EMD is one of the dog breeds that have a higher risk for EU (Fritsche *et al.* 2014). Out
59 of 552 classified EMD nearly half showed intravesicular ectopia, i.e. 130 females and 132
60 males. One fifth, i.e. 25 females and 84 males, had at least one extravesicular ectopic
61 termination. Urinary incontinence was a complaint in 3% and 27% of them. Hydronephrosis
62 and/or hydroureter was noticed by ultrasonography in 1% and 14% of the intravesicular and
63 extravesicular cases, respectively. In one third of them this was an incidental finding
64 (Fritsche *et al.* 2012). The breed predisposition indicates a genetic background, however
65 exaggerated breeding restrictions to reduce the risk of clinically affected dogs in the offspring
66 are of concern, as the EMD has already a high average inbreeding coefficient around 40%
67 (Schrack *et al.* 2017). Therefore, selection based on genotypes would be desirable, but first
68 attempts to associate five suitable candidate genes, selected from mouse studies (Uetani &
69 Bouchard 2009), to EU in EMD were unsuccessful (North *et al.* 2009). In humans, congenital
70 anomalies of the kidney and urinary tract (CAKUT) are a genetically heterogeneous group
71 of developmental disorders (syndromic and non-syndromic) with highly varying phenotypes.

72 Even though single gene mutations were shown to cause renal anomalies in mice, the
73 elucidation of CAKUT cases in humans remains difficult (Nicolau *et al.* 2016). Genetic
74 heterogeneity, modifier genes, allelic variation in gene expression, epigenetic effects,
75 complex modes of inheritance and environmental effects may hamper the clarification of the
76 genetic basis of sporadic and familial CAKUT (Yosypiv 2012).

77 Using complex segregation analysis, our group previously showed a genetic background for
78 EU in EMD and the possible involvement of a major gene for the EU-3 phenotype (Fritsche
79 *et al.* 2014), while the evaluation of an X-linked mode of inheritance, which could explain the
80 observed male predominance for extravesicular ectopia was not successful (Fritsche *et al.*
81 2014). The goal of the present study was to re-estimate the heritability for EU-3 and identify
82 genetic variants associated with EU and candidate genes within chromosomal regions of
83 such variants. Ureteric openings were determined for 1421 EMDs born between 1996 and
84 2016 and registered by official national kennel clubs. The majority of dogs were between six
85 and twelve months old when ultrasonographical screening was performed by authorized
86 private veterinarians and university clinics. This screening method was previously validated
87 by comparison with dissection (Balogh *et al.* 2015) and already used to elucidate the mode
88 of inheritance of EU by multivariate mixed logistic regression (Fritsche *et al.* 2014). All EU
89 diagnoses were finally assessed by one researcher in order to avoid a bias through the first
90 examiner. The dogs were classified based on the more caudally placed ureteral opening
91 into three phenotype classes, namely EU-1 (both ureters in the correct anatomical position,
92 i.e. a distance between the more caudal ureteral opening and the vesicourethral junction of
93 at least 1.8 cm (Balogh *et al.* 2015; Rozear & Tidwell 2003)), EU-2 (a distance of the more
94 caudal ureteral opening and the vesicoureteral junction between 1.1 and 0.1 cm) and EU-3
95 (at least one ureteral opening located extravesically in the urethra or genital tract). Dogs
96 which had not been examined or for which no conclusive diagnosis was attained, were
97 classified as EU-0. Pedigree information for the dogs was available through an in-house

98 EMD database, merging all EMDs into one single family. We re-estimated the narrow sense
99 heritability (h^2) of EU-3 under a threshold-liability model (Lee *et al.* 2011) using phenotypes
100 of 98 cases (73 males and 25 females) and 151 EU-1 controls (32 males and 119 females)
101 and pedigree information of 4522 dogs. The analysis included sex as a fixed effect. EU-3
102 was found to be heritable, with estimates of 0.713 and 0.960 in the 0-1 risk and liability
103 scales, respectively. These findings led us to hypothesize that a major risk locus may
104 underlie EU-3, which could be presumably detected through a genome-wide association
105 study (GWAS).

106 For the GWAS, genomic DNA was extracted from EDTA blood samples of 381 EMDs with
107 phenotype EU-1 (n=218), EU-2 (n=28) or EU-3 (n=135). Genotyping was performed using
108 the Illumina® CanineHD assay (Illumina Inc., San Diego CA, USA) at GeneSeek (part of
109 Neogen Corporation in Lincoln, USA). This chip contains approximately 173000 single
110 nucleotide polymorphism (SNP) markers distributed throughout the genome with an average
111 density of 70 markers per million base pairs, allowing for a robust within-breed association
112 analysis.

113 Due to the high selective pressure and inbreeding levels in EMD, our GWAS analysis
114 included dominance and autozygosity effects, apart from additive marker effects (see
115 supplementary material). The family-wise error rate was controlled by adopting a LD-
116 corrected Bonferroni significance level of $0.05/K$, where K is the effective number of
117 independent markers. The approach was similar to the simple method (Gao *et al.*, 2008),
118 except that K was estimated from the ratio between the total number of markers and the
119 average number of tag-partners per marker ($r^2 > 0.3$), instead of the eigenvalues required
120 to explain 99.5% of the variance in the genotype matrix. After scanning all autosomes (Fig.
121 1), we found six genome-wide significant loci ($p\text{-value} < 9.65 \times 10^{-5}$), which are presented in
122 Table 1.

123 Significant markers were observed in five regions on canine chromosomes (CFA) 3, 17, 27

124 and 30. On CFA 3, SNP BICF2P957732 is located near *MCTP2*, which encodes a
125 transmembrane protein with Ca^{++} binding domains involved in signal transduction or
126 membrane trafficking (Shin *et al.* 2005). Lalani and coworkers (2013) found gross heart
127 anomalies in *Xenopus* embryos associated with morpholino knockdown of *MCTP2*. The
128 SNP BICF2P527992 is in an intron of the IQ motif containing GTPase activating protein 1
129 gene (*IQGAP1*) and presented a significant dominance effect on the investigated trait.
130 *IQGAP1* is an interesting functional candidate gene because it is involved in the regulation
131 of the beta-catenin/GATA3 pathway in *Xenopus* embryos (Goto *et al.* 2013). The beta-
132 catenin/GATA3 pathway is involved in the formation of the ureteric bud, and loss of function
133 of *GATA3* leads to ectopic ureteric bud formation and severe urogenital abnormalities (Grote
134 *et al.* 2008). However, *lqgap1*-null mice did not show any observable phenotype (Li *et al.*
135 2000). There is no clear functional candidate gene for the association signal on CFA 17,
136 however, the underlying effects were due to autozygosity in this locus. The most intriguing
137 association signals are on CFA 27. There is one signal at ~1 Mb overlapping with the *HOXC*
138 gene cluster. *HOX* genes are obvious functional candidate genes for phenotypes that
139 involve potential defects in development (Mallo & Alonso 2013). The other association signal
140 at ~23 Mb on CFA 27 is located in an extremely gene poor region upstream of the *SOX5*
141 gene, which encodes another transcription factor involved in development. Coding variants
142 may lead to Lamb-Shaffer syndrome in humans, a neurodevelopmental disorder, sometimes
143 seen in combination with variable skeletal abnormalities (Nesbitt *et al.* 2015) but extremely
144 rarely with urogenital malformations (Lee *et al.* 2013). Therefore, it remains unclear whether
145 *SOX5* is the causative gene underlying this association signal. As the region of this
146 association signal is extremely gene poor, it might have important regulatory functions that
147 are not necessarily restricted to *SOX5* (Ovcharenko *et al.* 2005). The association signal on
148 CFA 30 is located between the *SMAD6* and *SMAD3* genes, encoding again important
149 transcription factors of the TGF-beta signaling pathway that also have a role in development

(Macias *et al.* 2015). While our GWAS failed to pick up any candidate genes known from human studies, it is remarkable that 3 of the 5 putative association signals fall near the genes for developmental transcription factors. As the p-values only just exceed the significance threshold, the findings need to be regarded with caution and the associations should be validated in a larger set of animals. In addition, nine genes which were previously shown to be involved in congenital anomalies of the lower urinary tract (CALUT) in humans (reviewed in Rasouly & Lu 2013) were analysed by comparing the genomes of 296 dogs of different breeds and eight EMD genomes with either EU-1 or EU-3 phenotypes. We did not find any protein-changing variants in these candidate genes (not shown).

Despite the high heritability found for ectopic ureters the association study failed to come up with a single strong association signal. There are at least two possible explanations for such a finding: (1) Despite the high heritability it is possible that many genetic risk loci with small effects are involved in the formation of EU. (2) It also cannot be excluded that a genetic variant, which predisposes the dogs for the formation of ectopic ureters, is fixed in the EMD population and therefore not detectable by the genome-wide association study.

In the latter scenario, modifier genes may still modulate the phenotype despite a unique underlying ectopic ureter genotype, similar to humans (Yosypiv 2012). This assumption is supported by the high average inbreeding coefficient of extant EMDs (Schrack *et al.* 2017), as well as by the much lower prevalence of ectopic ureters in the related Appenzeller mountain dog breed (Bitterli 2011). However, the high heritability seems to contradict this hypothesis. Even if we failed to identify strong candidates known from human studies, the signals seem to be quite clear for the relatively small number of animals, supporting an oligogenic inheritance. However, before using some of those SNPs for marker-assisted selection of breeding EMDs, further research is needed to support this data.

174

175 **Competing interests**

176 The authors declare that they do not have any competing interests.

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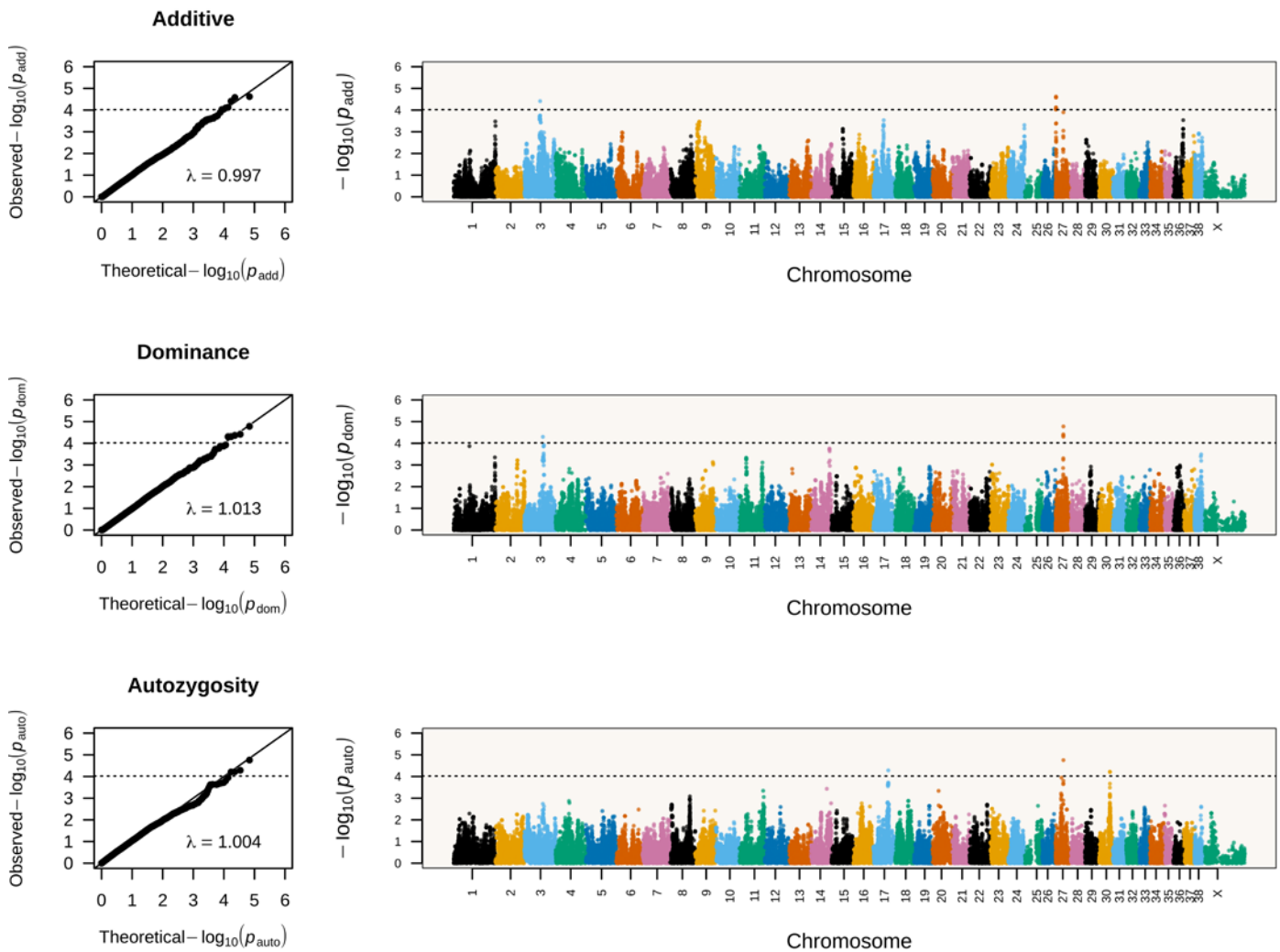
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316 **Figure 1 |** Quantile-quantile and Manhattan plots for additive (add), dominance (dom) and autozy-
317 gosity (auto) effects. The dashed horizontal line corresponds to the LD-adjusted Bonferroni thresh-
318 old ($p = 9.65 \times 10^{-5}$).

319 **Table 1.** Summary statistics for significant markers associated with ectopic ureters in Entlebucher mountain dogs

320

CFA ¹	Position ²	SNP ³	Position ³	⁴ Alleles	⁵ ♂ control	⁶ ♂ case	⁷ ♀ control	⁸ ♀ case	⁹ Inheritance model	¹⁰ P-value	¹¹ nearest gene	¹² Distance
3	45655143	BICF2P957732	45655143	A/C	23/9/0	39/27/6	95/22/2	9/13/3	Additive	3.89E-005	MCTP2	187,272
3	53778402	BICF2P527992	53778402	T/C	13/16/3	43/23/7	57/56/6	18/5/2	Dominance	5.05E-005	IQGAP1	0
17	43412582	BICF2P1360326	43412582	C/T	29/3/0	57/15/0	89/29/1	21/3/0	Autozygosity	5.23E-005	CTNNA2	59,764
27	1007730	TIGRP2P347803_rs8943392	1007730	T/C	20/12/0	65/8/0	95/23/1	24/1/0	Additive	9.38E-005	NEF2	0
27	1168218	BICF2S22927985	1168218	G/A	21/11/0	67/6/0	97/21/1	24/1/0	Additive	9.21E-005	HOXC4	41,292
27	1411816	BICF2G630137589	1411816	G/A	13/17/2	54/19/0	73/42/4	19/6/0	Additive	2.63E-005	HOXC13	88,346
27	1415865	BICF2G630137593	1415865	T/C	13/17/2	54/19/0	73/42/4	19/6/0	Additive	2.63E-005	HOXC13	109,704
27	1428628	BICF2G630137612	1428628	G/A	14/16/2	54/19/0	74/41/4	19/6/0	Additive	7.91E-005	HOXC13	12,763
27	1433130	BICF2G630137624	1433130	C/T	13/16/3	52/21/0	71/44/4	19/6/0	Additive	2.38E-005	HOXC13	17,265
27	1437735	BICF2G630137629	1437735	G/A	14/16/2	53/19/0	74/41/4	19/6/0	Additive	7.47E-005	HOXC13	21,870
27	22783765	BICF2G630149192	22783765	C/T	19/12/1	45/25/2	70/46/3	16/5/4	Dominance	4.39E-005	SOX5	73,896
27	22929398	BICF2P1108722	22929398	A/G	18/13/1	43/28/2	70/46/3	16/5/4	Dominance	3.88E-005	lncRNA	0
27	22994047	BICF2P830285	22994047	T/G	18/13/1	41/30/2	69/46/4	16/5/4	Dominance	5.00E-005	lncRNA	0
27	23114194	BICF2P595351	23114194	T/C	18/13/1	40/31/2	69/44/6	16/5/4	Dominance	1.67E-005	lncRNA	0
30	31140902	BICF2P906072	31140902	C/T	22/10/0	46/23/3	78/37/4	16/8/1	Autozygosity	6.06E-005	SMAD3	105,411
30	31236521	BICF2P664860	31236521	A/G	22/10/0	46/24/3	78/37/4	16/8/1	Autozygosity	6.23E-005	SMAD3	9,792

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322 ¹ Canine chromosome, ²SNP position in the corresponding chromosome (CanFam3.1 genome version) Annotation Release 103, ³SNPs in the
323 Illumina® CanineHD bead chip, ⁴minor allele/major allele, ⁵⁻⁸SNP genotype distribution, ⁹gene effect, ¹⁰P-value, ¹¹gene symbol of the nearest gene
324 of the reported SNP, ¹²Distance in bp between SNP and nearest gene (0=SNP within gene).